Site-directed mutagenesis of Tyr-189 and Lys-193 in NADPH: protochlorophyllide oxidoreductase from *Synechocystis*

D. J. Heyes[1] and C. N. Hunter

Krebs Institute for Biomolecular Research and Robert Hill Institute for Photosynthesis, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, U.K.

Abstract

NADPH:protochlorophyllide oxidoreductase (POR) catalyses the light-dependent reduction of protochlorophyllide to chlorophyllide, a key regulatory reaction in the chlorophyll biosynthetic pathway. Sequence comparisons have revealed that POR is a member of the short-chain alcohol dehydrogenase family of enzymes. A tyrosine and a lysine residue are conserved throughout all members of this family, and are proposed to be within the active site. This present study describes how site-directed mutagenesis has been used to change Tyr-189 to Phe and Lys-193 to Arg in the *Synechocystis* POR enzyme. The mutant enzymes were produced with a His tag in *Escherichia coli* and subsequently purified on a Ni^{2+}-affinity column. The two mutations resulted in inactive enzymes, indicating that both residues are crucial for activity. The K_d value for NADPH binding to the K193R mutant was significantly higher than for the wild-type enzyme, suggesting that the affinity for NADPH has also been reduced.

Introduction

The chlorophyll biosynthetic enzyme NADPH: protochlorophyllide oxidoreductase (POR; EC 1.3.1.33) catalyses the light-dependent reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) [1]. It is one of only two enzymes to require light for catalysis; the other is DNA photolyase [2]. As a result of its requirement for light, this reaction is an important regulatory step in the chlorophyll biosynthetic pathway and subsequent assembly of the photosynthetic apparatus [3].

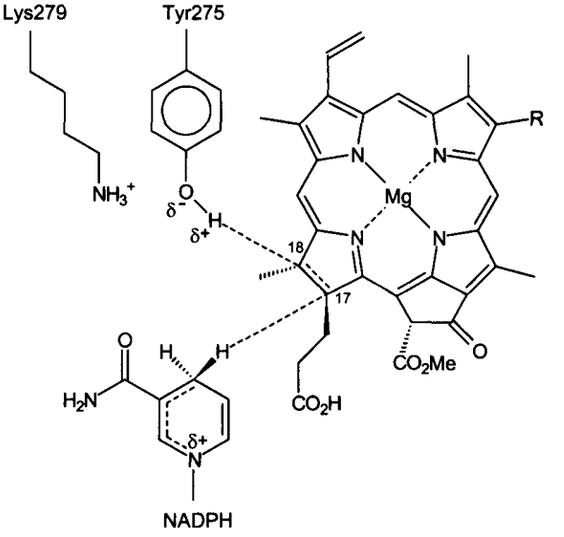
Sequence comparisons with human carbonyl reductase and pig 20β-hydroxysteroid dehydrogenase led to POR being classified as a member of the short-chain alcohol dehydrogenase family [4]. Members of this family have many structural features in common and have been used as a template to construct a homology model of POR [5]. A tyrosine and a lysine residue are absolutely conserved throughout all members of this family, and both residues are thought to be essential for catalysis [6]. The Tyr may be deprotonated, acting as a general acid to facilitate hydride transfer to or from NAD(P)/H [7]. The Lys residue is proposed to be important for lowering the apparent pK_a of the phenolic group of the Tyr, which is normally around 10, allowing deprotonation to occur. It may also play a role in ensuring that the nicotinamide ring of NAD(P)/H is in the correct orientation for *pro-S* hydride transfer [8]. Mutagenesis studies with human 15-hydroxyprostaglandin dehydrogenase [9], *Drosophila* alcohol dehydrogenase [10–12], rat 11β-hydroxysteroid dehydrogenase [13] and rat dihydropteridine reductase [14] have confirmed that these Tyr and Lys residues are important for catalysis.

The roles of these two residues in POR have also been studied [15,16]. The gene encoding the

Figure 1

Model of the proposed mechanism of catalysis of POR

The proton at the C-18 position of Pchlide is derived from Tyr-275 (numbering of pea POR), and the hydride transferred to the C-17 position is derived from the *pro-S* face of NADPH. R represents CH_2-CH_3 in monovinyl (MV)-Pchlide or CH=CH_2 in divinyl (DV)-Pchlide (drawn by Dr Helen Wilks).



Key words: chlorophyll biosynthesis, chlorophyllide, deprotonation, site-directed mutants.

Abbreviations used: POR, NADPH:protochlorophyllide oxidoreductase; Pchlide, protochlorophyllide; Chlide, chlorophyllide.

[1]To whom correspondence should be addressed (e-mail d.j.heyes@sheffield.ac.uk).

enzyme from pea was found to restore bacteriochlorophyll synthesis in *Rhodobacter capsulatus* strains harbouring a mutation in one of the three subunits of the light-independent Pchlide reductase. When Tyr-275 or Lys-279 was mutated, light-dependent complementation no longer occurred, suggesting that both residues are critical for POR activity [15]. A model for catalysis was proposed whereby the hydride transferred to the C-17 position of Pchlide is derived from the *pro-S* face of NADPH and the proton at the C-18 position is derived from Tyr-275 (Figure 1). Further studies on the pea enzyme have proposed that Tyr-275 and Lys-279 participate in the proper co-ordination of NADPH and Pchlide in the enzyme catalytic site [16]. This present work describes how the corresponding residues in the *Synechocystis* enzyme, Tyr-189 and Lys-193, have been mutated, and how the activity and NADPH-binding properties of the site-directed mutants have been analysed.

Materials and methods

His-tagged POR from *Synechocystis* sp. PCC6803 was overproduced in *Escherichia coli* and purified as described previously [17]. The oligonucleotides Y189F1 (5′-GGGCAAAGCG**TTT**AAAGATAGTAA-3′) and Y189F2 (5′-TTACTATCTTT-**AAA**CGCTTTGCCC-3′) were used to change Tyr-189 into Phe (altered codon is shown in bold). A second set of oligonucleotides, K193R1 (5′-TAAAGATAGTAGGCTCTGCAATAT-3′) and K193R2 (5′-ATATTGCAGAGCCTACTATCTTTA-3′), was used to change Lys-193 into Arg (altered codon is underlined). PCR reactions were performed in a total volume of 100 μl containing 200 μM dNTPs, 0.25 μM of each nucleotide primer, 2 ng of *Synechocystis* genomic DNA and 2.5 units of *Taq* DNA polymerase in 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001 % gelatin. The reactions were denatured for 10 min at 94 °C, followed by 40 cycles of amplification (94 °C, 45 s; 50 °C, 45 s; 72 °C, 90 s), and a final extension for 10 min at 72 °C in a Mastercycler 5330 (Eppendorf). The two resulting PCR products were cloned into the pET9-His vector and the enzymes were expressed and purified as described previously [17].

The activity of wild-type POR, and of the Y189F and K193R mutants, was measured as described previously [17] using 4 μM enzyme, 15 μM Pchlide and 10 μM NADPH. The fluorescence enhancement of NADPH that is observed upon binding to POR [17] was used to determine the apparent dissociation constant of NADPH binding to each enzyme.

Results

The site-directed mutants, Y189F and K193R, were produced by PCR and cloned into the pET9-His vector. Both inserts were sequenced and were found to contain the single correct codon change. Upon induction, synthesis of the His-tagged proteins wild-type POR, Y189F and K193R was observed. All proteins were purified using a single-step Ni^{2+}-affinity column, and were estimated to be greater than 90 % pure after this purification step (Figure 2).

No Chlide production was observed for either of the mutants, suggesting that both Y189F and K193R are inactive. The NADPH-binding properties of wild-type POR and the two mutants were determined using fluorescence analysis. All measurements were carried out using 5 μM protein to ensure that a direct comparison could be made between the enzymes. Figure 3 shows the fluorescence enhancement at each NADPH concentration caused by binding to wild-type POR, Y189F or K193R. K_d values were calculated to be 0.81 ± 0.17 μM for binding to wild-type POR, 1.40 ± 0.33 μM for binding to Y189F, and 24.4 ± 8.8 μM for binding to K193R.

Figure 2

SDS/PAGE analysis of purified wild-type POR, and of mutants Y189F and K193R

The SDS/PAGE gel shows wild-type POR, Y189F and K193R after purification on a Ni^{2+}-affinity column. Lanes 1 and 5 contain molecular mass standards (myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa); lane 2, purified Y189F; lane 3, purified K193R; lane 4, purified wild-type POR.

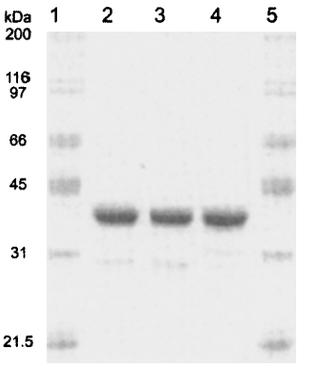
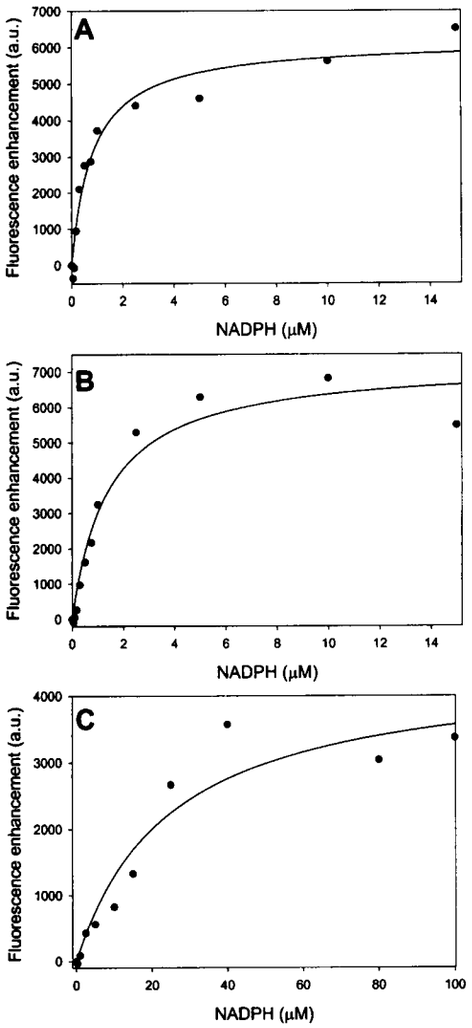


Figure 3

NADPH fluorescence enhancement upon binding to wild-type POR, and to mutants Y189F and K193R

The enhancement in NADPH fluorescence was measured upon binding to 5 μM wild-type POR (**A**), Y189F (**B**) or K193R (**C**) in assay buffer using a multi-well plate reader. The samples were excited with a wavelength of 360 nm through a 40 nm band pass, and fluorescence emission was recorded at 460 nm through a 40 nm band pass.



Discussion

A Tyr and Lys residue are absolutely conserved throughout all PORs, and indeed among all members of the short-chain alcohol dehydrogenase family [6,15]. Both residues have been implicated as being essential for catalysis, and this is supported by site-directed mutagenesis studies [15]: unlike wild-type POR, site-directed mutants were no longer able to complement Pchlide reduction mutants in *R. capsulatus*.

We recently described the production, kinetic characterization and NADPH-binding analysis of purified His-tagged POR [17]. In the present work, the corresponding residues in this enzyme, Tyr-189 and Lys-193, have been mutated to Phe and Arg respectively. Sequencing revealed that the *por* gene had been altered correctly, and in each case the recombinant enzyme was produced with a His tag and purified to homogeneity. Activity measurements showed that Pchlide reduction was totally abolished with both Y189F and K193R. This is in agreement with previous findings for alcohol dehydrogenase [10–12], 11β-hydroxysteroid dehydrogenase [13], 15-hydroxyprostaglandin dehydrogenase [9] and dihydropteridine reductase [14], where similar mutations to these two residues resulted in largely inactive enzymes. A model for POR catalysis has been suggested that involves the Tyr and Lys residues in the reaction mechanism [15]. The proton at the C-18 position of Pchlide is derived from the Tyr, whereas the hydride transferred to the C-17 position is derived from the *pro-S* face of NADPH. The close proximity of the Lys residue is necessary to lower the pK_a of the Tyr in order to facilitate deprotonation of the phenolic group (Figure 1). The results described here for *Synechocystis* POR would appear to support this model and to show the importance of these residues for activity.

As both residues are implicated in catalysis, it was interesting to see if the mutations had any effect on the NADPH-binding properties of the enzyme. The K_d value for NADPH binding to Y189F was found to be only slightly higher than that obtained for wild-type POR, suggesting that NADPH binding had not been significantly affected. This is not surprising, as Tyr and Phe have a similar structure and therefore the mutation would not be expected to cause any major perturbations to the NADPH-binding pocket. However, the K_d value obtained for K193R was more than 25-fold higher than that calculated for NADPH binding to the wild type. This indicates that swapping Lys-193 for Arg causes sufficient changes to lower the affinity of the enzyme for NADPH. Although both residues are positively charged, Arg is a considerably larger residue than Lys, which may result in the exclusion of NADPH from the binding pocket. The decrease in affinity for NADPH may also be explained by the other suggested role of the Lys residue, i.e. ensuring that the nicotinamide ring of NAD(P)/H is in the correct orientation for *pro-S* hydride transfer [8].

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Making light of it: the role of plant haem oxygenases in phytochrome chromophore synthesis

M. J. Terry*[1], P. J. Linley* and T. Kohchi†
*School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K., and †Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan

Abstract

The haem oxygenase (HO) enzyme catalyses the oxidation of haem to biliverdin IXα, CO and Fe^{2+}, and performs a wide variety of roles in Nature, including degradation of haem from haemoglobin, iron acquisition and phycobilin biosynthesis. In plants, HOs are required for the synthesis of the chromophore of the phytochrome family of photoreceptors. There are four *HO* genes in the *Arabidopsis* genome. Analysis of a mutant deficient in HO1 (the *hy1* mutant) has demonstrated that this plastid-localized protein is the major HO in the phytochrome chromophore synthesis pathway. HO2 may also have a minor role in this pathway, but our understanding of the divergent roles of this small gene family is still far from complete.

Key words: biliverdin, chloroplasts, light and plastid signalling, photoreceptor, tetrapyrroles.

Abbreviations used: BV, biliverdin; EST, expressed sequence tag; HO, haem oxygenase protein; *HO*, haem oxygenase gene; PΦB, phytochromobilin.

[1]To whom correspondence should be addressed (e-mail mjt@soton.ac.uk).

Haem oxygenase (HO) – a universal enzyme for haem degradation

HO was originally identified as a key enzyme in the degradation of haem through its role in bilirubin production in rat liver [1], and is still the only enzyme known that can catalyse haem degradation. As shown in Scheme 1, HO oxidizes haem at the α-*meso* position to give biliverdin (BV) IXα, Fe^{2+} and CO in a reaction requiring molecular oxygen and electrons from NADPH. The mechanism of this reaction has been described in detail recently, and proceeds through a number of relatively stable intermediates, including α-*meso* hydroxyhaem and verdohaem [2]. As discussed below, additional proteins are required to utilize NADPH, and the type of protein used is species-dependent: mammalian HOs require cytochrome P450 reductase [2], while bacterial HOs have been shown to use ferredoxin [3,4] or putidaredoxin [5] together with an accompanying NAD(P)H-dependent reductase.

While haem degradation is a common feature of all HOs, this enzyme actually has a great variety of roles in Nature, due in part to the diversity